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Lipids modulate the insertion and folding of the nascent chains of alpha helical membrane proteins

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Abstract

Membrane proteins must be inserted into a membrane and folded into their correct structure in order to function correctly. This insertion occurs during translation and synthesis by the ribosome for most α-helical membrane proteins. Precisely how this co-translational insertion and folding occurs, and the role played by the surrounding lipids, is still not understood. Most of the work on the influence of the lipid environment on folding and insertion has focussed on denatured, fully translated proteins, and thus does not replicate folding during unidirectional elongation of nascent chains that occurs in the cell. This review aims to highlight recent advances in elucidating lipid composition and bilayer properties optimal for insertion and folding of nascent chains in the membrane, and in the assembly of oligomeric proteins.

Introduction

Membrane proteins comprise over a third of proteins in the cell, and yet remain poorly understood. Transmembrane (TM) α-helical proteins reside within a lipid bilayer, and thus depend upon the surrounding lipids in order to fold and function correctly. α-helical proteins are typically inserted into the lipid membrane co-translationally via an insertase, such as the *E. coli* translocon SecYEG [1, 2] or via YidC [3]. To date, the majority of work on the effect of lipids on TM protein folding and function has used fully translated protein [4-13], and thus cannot be generalised to the insertion of nascent chains as they emerge from the ribosome (**Fig. 1**). The aim of this paper is to discuss the effects of lipid properties on nascent chain folding in the absence of cellular insertases. In particular, this paper will focus on larger and oligomeric proteins in planar bilayer mimetics.

Why study nascent chain folding?

The majority of work on membrane protein folding uses relatively well-behaved proteins that have been purified following cellular overexpression and partially unfolded in a denaturant. A significant amount of structure remains in this unfolded state [9, 11, 14-20]. While still providing valuable information, particularly for example with regard to thermodynamics, it is unclear precisely how this *in vitro* reversible folding relates to co-translational folding in the cell, in which insertion and folding occur during polypeptide synthesis (**Fig. 1**)[21-23].

Studies of nascent chain insertion and folding often use membrane extracts, such as *E. coli* inner membrane vesicles [24-27], and microsomes (often extracted from dog pancreas) [28-31]. These extracts are useful as they include all the integral membrane apparatus required to aid insertion of proteins into the bilayer. Much work has focussed on the insertion of model fusion proteins and truncated or stalled polypeptides [32-36]. These studies are valuable, as they provide tractable experimental systems that aid understanding of insertion of key protein segments and indicate how translocon assisted insertion may occur. They do not however aid understanding of how global membrane properties such as lateral pressure, headgroup charge and lipid phase govern insertion and folding.

Nascent chain folding *in vivo*

In vivo, membrane proteins are inserted into the bilayer via SecYEG/Sec61, or via YidC-like insertases [37-39]. The majority of α-helical membrane proteins insert into the membrane co-translationally [21-23]. Those that insert co-translationally via the Sec pathway first interact with the Signal Recognition Particle (SRP) upon emergence from the ribosome, and are chaperoned to the membrane for insertion via interaction with the SRP receptor, FtsY [40-45]. In the standard model

for translocon guided insertion, the nascent chain emerges from the ribosome directly into the translocon channel, and the TM helices partition into the membrane via a lateral gate in the translocon. A recently proposed alternative model [38] (**Fig. 2**) suggests that the translocon acts as a chaperone for polar loops to traverse the membrane, and the TM helices do not enter the translocon channel. Instead, TM helices first partition with the interfacial headgroup region of the lipid bilayer, before inserting across the bilayer by sliding down the translocon lateral gate. In this model the TM helices are in constant contact with the lipid bilayer, and use the highly favourable association with the hydrophobic interior of the membrane as a driving force for insertion [38]. Thus, the study of membrane protein insertion in the absence of translocon is informative, as not only could the initial interaction with the lipid bilayer be an important step during insertion, but if folding is thermodynamically driven then the partitioning free energies are independent of an insertase and the pathway taken.

YidC-like insertases include YidC of *E. coli*, Oxa1 of mitochondria and Alb3/Alb4 of chloroplast thylakoids [46]. The mechanisms of insertion via these YidC-like insertases are less well understood. To date there are only a small number of substrates known to be dependent on the *E. coli* YidC, including the homopentameric channel MscL [37, 46-48]. Substrates for YidC are thought to be small proteins with one or two membrane spanning regions [37]. YidC is thought to have a role in assembly of large membrane complexes, as YidC downregulation affects the assembly of a number of respiratory complexes, such as the F_1F_0 ATP synthase [37, 46]. It is unclear whether this is because the complexes contain a YidC substrate which can no longer insert into the membrane, or because YidC has a direct role in membrane complex assembly.

Lipids modulate folding and insertion of membrane proteins

Membrane proteins reside in a lipid bilayer and are therefore constantly influenced by their lipid environment. The lipid composition in native membranes is remarkably different between different organisms, and even between organelles [49-52]. The *E.coli* inner membrane for example contains mainly unsaturated lipid chains with a high percentage of PE headgroups along with PG and cardiolipin [49, 52]. In contrast the plasma membrane of mammalian cells contains PC, PI, and cholesterol [50, 51], and is thicker than the *E.coli* inner membrane as it contains sphingolipids with long, saturated chains (C16-C32 chains, [50]). Given how varied native membranes are, replicating the complex native membrane for *in vitro* experiments is incredibly challenging.

The surrounding lipids can influence membrane proteins in a number of ways - their folding [6, 8, 10, 11], their insertion into the bilayer [4, 5, 7, 13, 16], their function [10, 53-57], their topology [58-67], and their oligomerisation [68-70]. α-helical membrane proteins are almost always inserted into the

bilayer co-translationally [21-23]; the lipid environment is therefore likely to be highly influential on the folding and insertion on the nascent chain as it emerges from the ribosome and translocon.

Lipids can influence membrane proteins directly via specific interactions with the headgroup region, or indirectly via the lateral pressure profile and the lipid phase. Lipid headgroups can be charged (e.g. PG, PS), or zwitterionic (e.g. PC, PE), and some (e.g. PC, PE) can hydrogen bond with other lipid headgroups, the aqueous phase or with membrane proteins [71-73]. Lipid chains which are saturated (e.g. DMPC, DPPC) have low lateral chain pressure, while introducing unsaturation to the lipid chains produces a more fluid bilayer and increases lateral chain pressure (e.g. DOPC) [49]. A further increase in lateral chain pressure can be produced by addition of the non-bilayer forming lipid PE. The small headgroups of PE cause negative curvature towards the aqueous phase, resulting in the lipid chains becoming constrained in the bilayer when mixed with a bilayer forming lipid such as PC [74]. The bilayer forming negatively charged lipid PG also has a slightly higher tendency to curve towards the aqueous phase than PC, particularly when there is a high concentration of divalent cations in the aqueous phase (as is the case during cell-free reactions) [10, 75]. While a high lateral chain pressure has been shown to hinder insertion [4, 5, 12, 13], there is a compensating decrease in headgroup pressure, which may aid association of TM helices with the bilayer headgroup region [4-6] (**Fig. 3**). Lipid phase is also an important property of lipid bilayers; lipids at gel phase likely hinder insertion (e.g. DMPC below 25 °C).

Lipids and cell-free expression of membrane proteins

The membrane mimetic supplied during the cell-free synthesis of membrane proteins has a significant effect on the yield of folded and functional protein produced, and has been the subject of multiple reviews [76-79]. It has been suggested that membrane proteins can fold correctly in a range of detergents or amphipols, and that the final folded state is independent of the folding route taken [80]. While this is true in some cases, the majority of membrane proteins do have specific membrane mimetic requirements to be folded and stable. Almost all previous work on the production of membrane proteins by cell-free expression therefore includes some degree of optimisation of the lipid and/or detergent environment [81-89]. This optimisation is often performed with the aim of producing as much functional protein as possible to do structural and characterisation studies on the final folded protein. Not many studies have systematically studied how different lipids and lipid properties affect the co-translational nascent chain folding process itself. Cell-free expression in the presence of either inner membrane vesicles [24-27] or microsomes [28-31] is common, and while able to mimic native membrane compositions does not offer insight into which lipid properties specifically influence folding and insertion. Studies which aim to look at

specific lipid properties have used defined mixtures of synthetic lipids to enable control over bilayer composition. Synthetic mixtures often include PC, which is not native to *E.coli* but readily forms well-characterised, unilamellar fluid bilayers to which other lipid types are added.

A hindrance in studying the effect of lipid properties on TM insertion is the tendency for liposomes to precipitate in cell-free reactions [76, 79] due to the high concentration of divalent cations present in many cell-free extracts. Many membrane proteins are therefore produced cell-free with PC lipids only, often extracted from soy bean, which are less prone to precipitation. Recent work has avoided this issue by using nanodiscs rather than liposomes to investigate lipid effects. Nanodiscs are composed of a small planar phospholipid bilayer surrounded by Membrane Scaffold Protein (MSP), and are around 10 nm in diameter. Care should be taken when comparing the results between nanodiscs and liposomes however, as the lipids in each have different global properties. Nanodiscs have altered phase behaviour compared to liposomes [90-92]. For example, DMPC has a significantly broader gel to liquid crystalline phase transition temperature in nanodiscs (between 20 - 40 °C) than DMPC in liposomes [90]**.**

Optimising the surrounding lipids promotes nascent chain folding

While there are very few studies on the effect of the lipids on nascent chain folding, a trend is beginning to emerge in which headgroup charge and chain unsaturation favour insertion and folding. Work on the *E. coli* rhomboid protease GlpG found that increasing the headgroup charge and lateral chain pressure via addition of DOPG and DOPE promoted spontaneous insertion, with the highest amount of insertion observed in 50:50 DOPE:DOPG and *E. coli* polar extract, and the lowest amount of insertion observed in DMPC [16]. The lipids in this study were supplied as liposomes to the PURExpress cell-free expression system, which has fewer problems with liposome precipitation than cell free extracts [16]. Work on the β1-adrenergic receptor (β1-AR) produced by an *E. coli* cell lysate (S30 extract) in the presence of nanodiscs has concluded that insertion of active protein into nanodiscs increases when unsaturation is introduced to the lipid chains, with nanodiscs composed of DOPC an improvement on those formed from DMPC. β1-AR also preferred charged headgroups, with both PG and PS favoured over PC headgroups. There was a further preference for *trans* lipid tails (DEPG) over *cis* lipid tails (DOPG). This study also found that most of the lipids tested produced around the same amount of inserted protein, but did not all result in active receptor [93], demonstrating the importance of testing proteins for function once they are inserted into a bilayer. Cell wall synthesis enzyme MraY from *E. coli* (EC-MraY) was found to prefer nanodiscs composed of DMPG to DMPC, and when a mixture of these two lipids was used, a minimum of 50 % DMPG was

required for insertion and activity. In contrast, MraY from *B subtilis* (BS-MraY) did not have a preference for a particular headgroup, despite being a homologous protein [94].

While there is an apparent trend emerging for a preference for charged headgroups and lateral chain pressure (**Table 1**), it is not universal. The *E. coli* disulphide bond reducing protein DsbB has been found to insert best into liposomes composed of 100 % DMPC, with a reduced insertion yield in the other lipids tested [16]. It could be expected that proteins from the same organism would have a preference for similar lipids, but this is clearly not the case. Studies from chemically denatured membrane proteins have indicated that the different lipid dependences of proteins may reflect contrasting folding mechanisms and rate-limiting steps. Differing aspects of membrane proteins, including folding rate, yield, topology and stability have dissimilar dependences on lipid headgroup charge and lateral pressure.

Making oligomeric membrane proteins using cell-free expression

Many membrane proteins function as a part of multi-subunit complexes *in vivo*, either as homo- or hetero-oligomers. However, it is difficult to express oligomeric proteins *in vitro*, as the individual subunits need to be either co-expressed and assembled prior to purification, or expressed and purified separately and then assembled. The route taken depends on whether assembly and folding are cooperative or independent [95, 96], and on how stable the individual subunits are. The use of cell-free expression can aid studies of oligomeric proteins, as it may be easier for individual subunits to interact in the cell-free reaction. *In vivo*, YidC-like insertases are thought to have a role in the assembly of large membrane complexes [37, 47, 48].

There are a small number of studies which have produced oligomeric proteins cell-free, and even fewer which have studied the optimum lipid composition for correct assembly. SecYEG has been synthesised cell-free in PUREfrex with soy-PC liposomes. In this study, the DNA template of each was titrated to find the DNA concentration required to express a 1:1:1 ratio of SecY, E and G. The resulting complexes were analysed by blue native PAGE, and were also assayed for translocation and insertion activity [97]. It was also found that a complex cannot be formed when SecE was omitted from the cell-free reaction, suggesting that SecY and E need to interact with each other before SecG.

The oligomeric states of EmrE, KcsA, proteorhodopsin, MraY and the voltage-sensing domain of the human proton channel (hHv1-VSD) when inserted into DMPC or DMPG nanodiscs has been studied by LILBID-MS (laser-induced liquid bead ion desorption mass spectrometry) [69, 70]. LILBID-MS is a recently developed technique which can resolve the oligomeric state of intact complexes in nanodiscs when made by cell-free expression. It was found that there was a degree of cooperativity

in the assembly of each oligomer. Proteorhodopsin in particular was found to contain primarily oligomers when a large excess of empty nanodiscs was present, and KcsA contained exclusively tetramers when the KcsA:nanodisc ratio was 2.5:1, indicating cooperativity in tetramer assembly [69].

LILBID-MS can provide valuable insight into how the lipid composition provided during cell-free synthesis can affect the oligomerisation of different membrane proteins. It was found that the oligomeric state of BS-MraY is the same in nanodiscs of either DMPC or DMPG, but EC-MraY is dimeric in DMPG and monomeric in DMPC [69], correlating with the functional data discussed above [94]. A 1:1 ratio of DMPC:DMPG contained 72 % dimers [69].

Cell-free expression of the homopentameric channel MscL

There are few oligomeric proteins which are as well-studied as the homopentameric mechanosensitive channel MscL. MscL is well characterised in terms of which lipids promote channel opening and function [13, 98], and has been shown by numerous groups to insert independently of the Sec translocon [47, 99, 100]. MscL is thought to be a substrate for YidC, but there is some uncertainty over whether YidC is required for insertion of the monomer [99] or assembly of the pentamer [47], or neither [100]. It has been found that the insertion of the MscL monomer decreased upon SRP depletion [47], indicating that the SRP is required for co-translational insertion of MscL. The majority of studies on the insertion pathway of MscL have been done in insertase-deficient cell lines. This *in vivo* work on MscL and the fact that it is a homopentamer makes it a good candidate to study oligomerisation via cell-free methods *in vitro*.

MscL has been synthesised cell-free in *E. coli* lysates in the presence of detergents [101, 102], non-detergent surfactants [103] and in soy-PC liposomes, in which patch clamping measurements indicated that the spontaneously inserted MscL was functional [100]. There are also studies on which lipids aid insertion of MscL into the bilayer when produced by cell-free expression. MscL has been expressed with commercial and in-house *E. coli* lysates in various lipid compositions [79, 100]. This work concluded that the fusion protein MscL-GFP preferred the unsaturated chains of DOPC to the saturated chains of DMPC and DPPC. The insertion yield of MscL (without GFP) produced cell free by an *E. coli* cell lysate in the presence of liposomes increased through addition of negatively charged DOPG. Addition of DOPE, which increases chain unsaturation and lateral chain pressure, increased the yield of inserted protein from around 20 % in DOPC to around 50 % in 1:1 DOPC:DOPE (**Fig. 3**). The highest insertion yield was observed in 100 % DOPG, where almost 80 % of the synthesised MscL inserts into the liposomes, showing a strong preference for negatively charged

headgroups. DOPG also has a slightly higher lateral chain pressure than DOPC due to its negative headgroup charge [10, 75].

As MscL functions as a homopentamer, the presence of MscL pentamer can be taken as an indication of correct assembly. MscL pentamers can be observed in 100% DOPG, but not monomers, demonstrating that the high amount of insertion observed in DOPG can be attributed to formation of pentamer (**Fig 3**). Oligomerisation in synthetic bilayers *in vitro* however may not necessarily represent oligomerisation *in vivo*, as a less crowded bilayer may enable insertion and pentamer formation in the absence of an insertase.

Conclusions

By the handful of studies conducted so far, it seems to be the case that insertion in the absence of a translocon is most efficient when association with the lipid headgroups is favourable (**Table 1**). This highly favourable headgroup association compensates for the high lateral pressures in the lipid chain region. This preference for a favourable lipid headgroup association for efficient insertion is supported by the recent model proposed for translocon assisted insertion ([38], **Fig. 2**), in which individual TM helices partition with the lipid headgroups before inserting across the bilayer. While it seems counterintuitive that a high lateral chain pressure aids insertion, it may be that the rate-limiting step is association with the headgroups, which is compensated for by adding lipids with high chain pressure and low lateral pressure in the headgroup region [7, 8, 104].

Different lipid preferences have been found for proteins from the same organism (e.g. GlpG and DsbB), and for homologues from different organisms (e.g. MraY from *E. coli* and *B. subtilis*). This is because the folding rate, yield, topology and stability of different membrane proteins have dissimilar dependences on lipid headgroup charge and lateral pressure [4-8, 10, 105], and are therefore influenced by lipid properties differently. Significantly more information is needed before we can truly understand how lipid properties will affect the folding and insertion of different nascent membrane proteins. There are other considerations which must be taken into account when optimising a bilayer for insertion. For example, addition of non-native PC lipids when studying *E.coli* proteins is often useful, as PC is highly amenable for *in vitro* use due to its fluid, unilamellar bilayer properties. Many membrane proteins are also dependent on specific lipids for function [10, 53-56], and these may not be the optimum lipids required for insertion and correct folding. An appropriate balance therefore needs to be made between lipids optimum for function, insertion and correct folding, and also for ease of use *in vitro*. The promising developments of both LILBID-MS [69, 70] and time-resolved surface-enhanced infrared spectroscopy [16, 106] in combination with nanodiscs of

defined lipid composition represent a significant step forward in aiding studies of nascent chain folding, oligomerisation and the influence of the surrounding lipids.

Abbreviations

PC, phosphocholine; PE, phosphoethanolamine; PG, phosphoglycerol; PI, phosphoinositol; PS, phosphoserine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 PC); DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (14:0 PG); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16:0 PC); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1 (Δ9-Cis) PC); DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1 (Δ9-Cis) PE); DOPG, 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (18:1 (Δ9-Cis) PG); DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (18:1 (Δ9-Cis) PS); DEPG, 1,2-dielaidoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (18:1 (Δ9-Trans) PS)

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Competing interests

The authors declare that there are no competing interests associated with this manuscript

Figure legends

Fig. 1 *In vitro* folding vs. nascent chain folding

Folding is often measured *in vitro* as shown in (**A**), where fully translated purified protein in detergent is reversibly unfolded in a denaturant. This unfolded state contains a high amount of residual helical structure. However folding in the cell occurs as the nascent chain is translated and inserted into the bilayer, shown in (**B**), where helices can interact with each other and with the membrane before translation is complete (ribosome not shown)

Fig. 2 Alternative models of insertion via SecYEG

(**A**) In the standard model of TM helix insertion, the TM helices emerge from the ribosome-SRP complex and enter the translocon channel. The hydrophobic TM helices partition into the membrane via the lateral gate of SecYEG. (**B**) In the alternative model proposed in [38], the TM helices associate with the headgroup interfacial region of the bilayer before inserting across the bilayer by sliding down the lateral gate in SecYEG. The helices therefore avoid being in contact with the hydrophilic interior of SecYEG. This hydrophilic interior provides a route for the periplasmic loops to cross the membrane. Larger periplasmic domains would also traverse the membrane in this way. A similar mechanism has been proposed for insertion via YidC-like insertases [37, 107].

Fig. 3 Lipid bilayers affect insertion of TM helices

(**A**) Bilayers with saturated chains, such as those in DMPC, have a fluid bilayer with low lateral pressure in the chain region, and high pressure in the headgroup region. Unsaturation in the lipid chains (DOPC) increases the lateral chain pressure, as does adding a non-bilayer forming lipid such as DOPE. (**B**) Bilayers with high lateral chain pressure have a corresponding low headgroup pressure, aiding TM helix association with the headgroup region. High lateral chain pressure does not prevent TM insertion across a bilayer, but can hinder it. A TM helix can insert more easily across a bilayer with low lateral chain pressure [104]. (**C**) MscL insertion approximately doubles when either PG or PE headgroups are added at a 1:1 ratio to PC. Insertion into liposomes composed of 100 % DOPG is 4 times higher than liposomes of 100 % DOPC, demonstrating a strong preference for charged headgroups. MscL was made using an S30 cell lysate following the procedures described in [108], and analysed and quantified by [^{35}S]Met incorporation as described in [16] Error bars are the SEM from 8 repeats. (**D**) MscL was made by cell-free expression using an S30 cell lysate in the presence of 100 % DOPG liposomes and analysed by DDM-PAGE, in which MscL pentamers remain intact [13].

Cell-free insertion into DOPG liposomes produces oligomeric MscL, and no detectable monomer (~ 15 kDa).

Protein		Mimetic	Effect	Ref
BS-MraY	10 TM Dimeric enzyme for cell wall synthesis	Nanodiscs	No preference for **DMPC** or **DMPG**	[69, 94]
DsbB	4 TM Disulphide bond reducing enzyme	Liposomes	Prefers low lateral chain pressure and neutral headgroups – **DMPC**	[16]
EC-MraY	10 TM Dimeric enzyme for cell wall synthesis	Nanodiscs	Prefers **DMPG** to DMPC, needs 50 % PG to function and form dimers	[69, 94]
β1-AR	7 TM GPCR	Nanodiscs	Prefers high lateral chain pressure and negative charge – **PG** or **PS** with **unsaturated *trans* chains**	[93]
GlpG	6 TM Rhomboid protease	Liposomes	Prefers high lateral chain pressure and negative charge – **DOPG** and **DOPE**	[16]
MscL	2 TM Pentameric mechanosensitive channel	Liposomes	Prefers high lateral chain pressure and negative charge – **DOPG** and **DOPE**	[79]

Table 1- Summary of optimum lipids for insertion and folding

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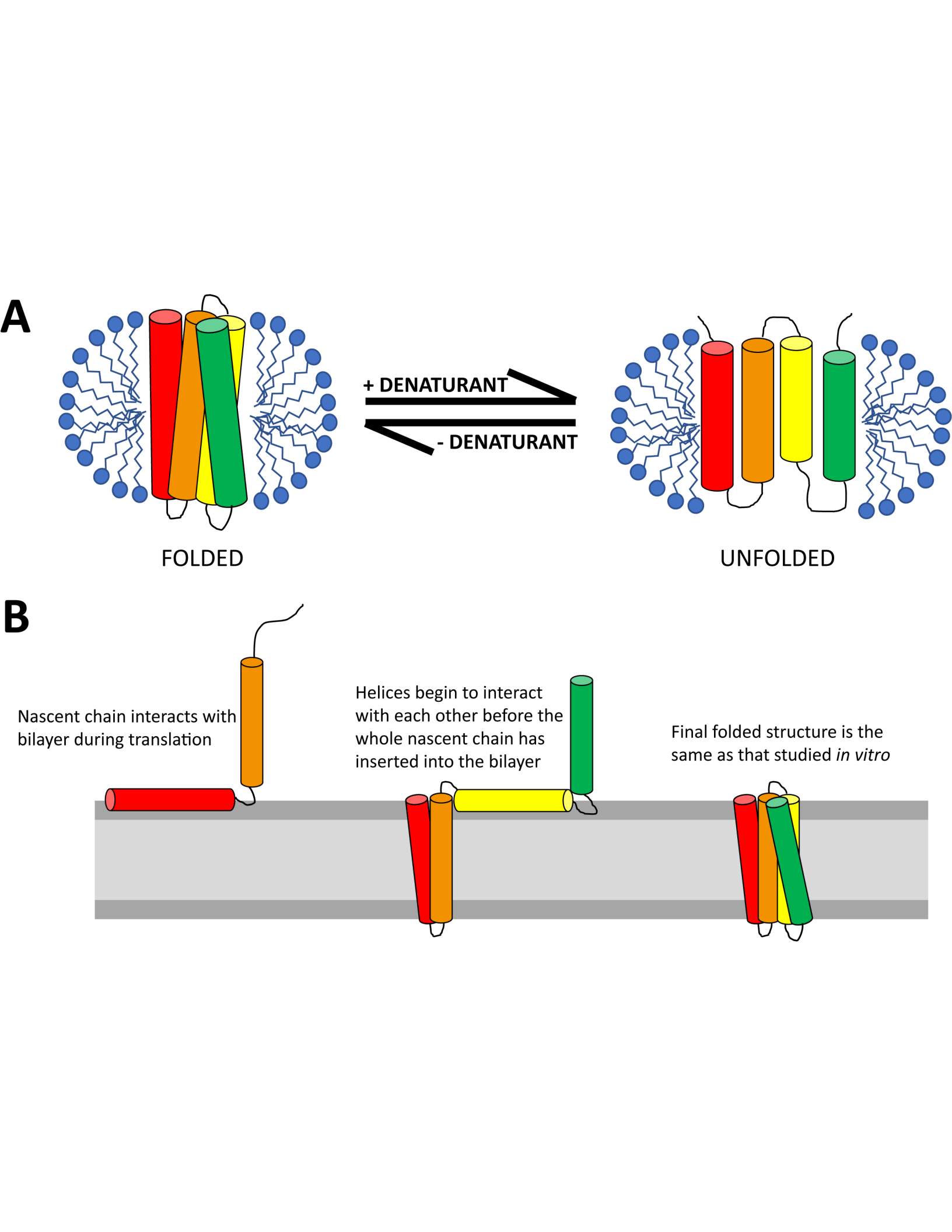
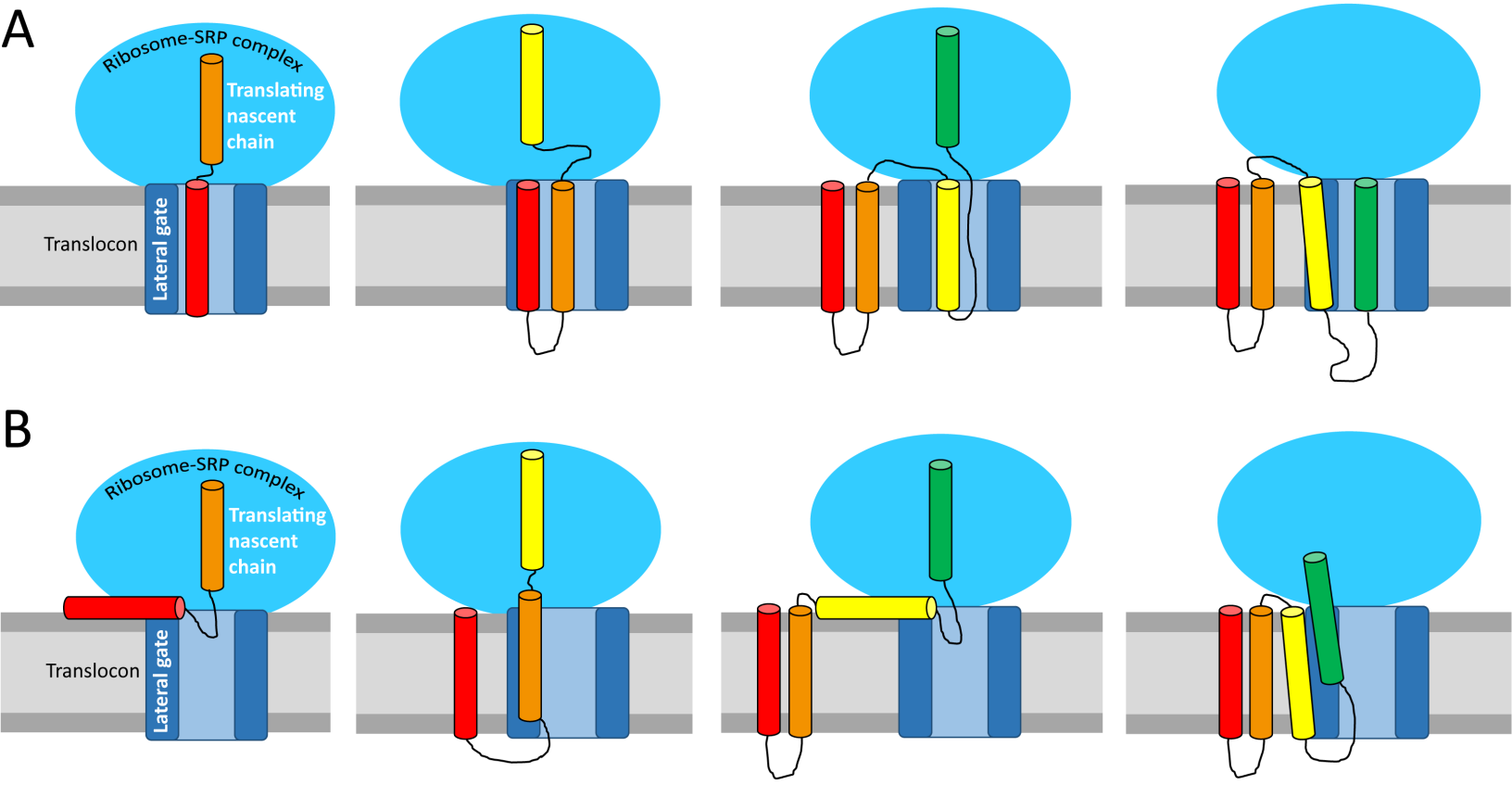
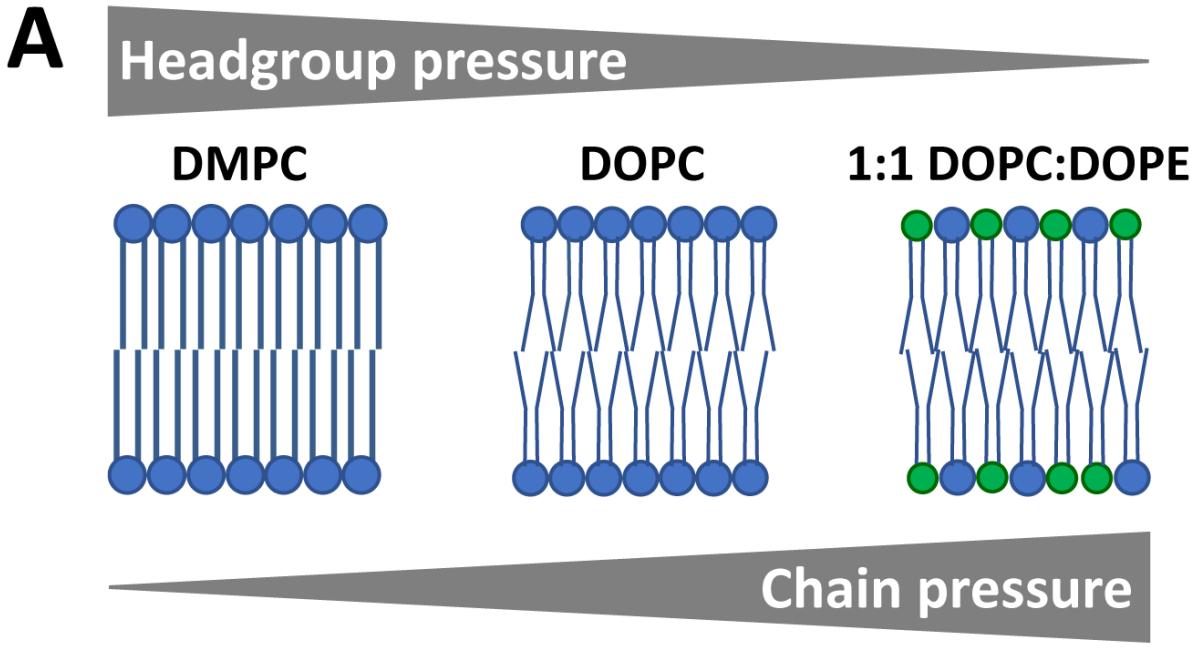
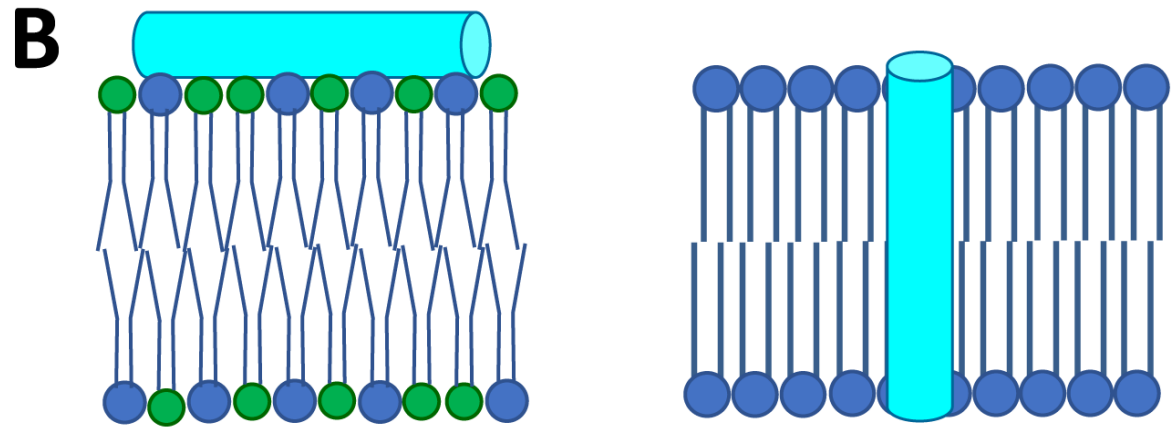

A
+ DENATURANT
- DENATURANT
FOLDED
UNFOLDED
B
Nascent chain interacts with bilayer during translation
Helices begin to interact with each other before the whole nascent chain has inserted into the bilayer
Final folded structure is the same as that studied *in vitro*


A
Ribosome-SRP complex
Translating nascent chain
Translocon
Lateral gate
B
Ribosome-SRP complex
Translating nascent chain
Translocon
Lateral gate





HIGH CHAIN PRESSURE. LOW HEADGROUP PRESSURE
TM ASSOCIATION WITH HEAD-GROUP IS FAVOURED

LOW CHAIN PRESSURE. HIGH HEADGROUP PRESSURE
TM INSERTION ACROSS THE BILAYER IS FAVOURED